Studies to Investigate the Effect of Co-targeting EGFR and c-MET Signaling Pathways in Esophageal Cancers

PhD Synopsis

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1. Background

Esophageal cancer (EC) is a life threatening disease with an incidence of 572034 new cases and (508585) mortalities annually worldwide. Esophageal cancer is the 7th most day-to-day malignancy and the 6th and 4th leading cause of death worldwide and India respectively (Ferlay *etal*., 2019). Based upon different etiological and pathological characteristics, EC has two main types, i.e. Esophageal Squamous Cell Carcinoma (ESCC) and Esophageal Adenocarcinoma (ADC). Despite significant advances in surgery and chemotherapy (Carboplatin, Paclitaxel, 5FU, Cisplatin) or targeted therapy (TT) (anti-EGFR) based chemo radiation therapy (CRT), EC is stillcharacterized by poor prognosis and low survival rates (5 year survival is 15-20%) (Pennathur *et al.,* 2013), primarily due to loco-regional/distant metastasis and failure to the currently available CRT's. The reasons of this poor response include inherit and acquired resistance to the CRT/TT treatment and associated severe toxicities resulting in significant co-morbidities (Rivelli *et al*., 2015). While the underlying mechanism of resistance to CRT is multifactorial, the recent molecular studies have shown the deregulated expression of drug targets, pro-survival and apoptotic pathway proteins by tumor cells (Thariat *et al*., 2007). Concurrent expression of EGFR and c-MET plays an important role in EC pathogenesis. It has been observed that EC patients with concurrent overexpression of EGFR and c-Met show notably worse survival than those that display overexpression of either of the two proteins individually (Ya-ping *et al*., 2016). Unfortunately, use of EGFR inhibitors as monotherapy has yielded modest benefits, often associated with the development of resistance and refractory tumors (Ayyappan *et al*., 2013 & Ku Gy *et al*., 2013). It has been observed that C-Met over-expression plays a key oncogenic role in about 35% of ADC (Ildiko Mesteri *et al*., 2014). The Interactions between EGFR and c-Met may decrease the effectiveness of anti-EGFR-targeted therapy. Activation of c-Met in the setting of EGFR blockade may be contributing to the limited clinical responses of EGFR inhibitors (Xu *et al*., 2015). The study has also been conducted in ESCC patients treated with anti EGFR drugs where it has been found that ESCC patients with low expression of c-MET had better Overall Survival (OS) than patients with overexpression of c-MET (Sheridan *et al*., 2014).

Afatinib, N-[4-[(3-chloro-4-fluorophenyl) amino]-7-[[(3S)-tetrahydro-3-furanyl]oxy]-6-quinazolinyl]-4-(dimethylamino)-2-butenamide]) is a second generation FDA approved

irreversible, tyrosine kinase inhibitor of all ErbB family members. Many preclinical (Mihali & Gyorffy, 2013) and clinical (Walter *et al*., 2013 and Ferrato & Gold, 2014) studies have shown that afatinib significantly inhibit growth of many cancers overexpressing either wild type EGFR and/or HER2 or EGFR L858R/T790M double mutations. Afatinib is a potent, orally administered, and irreversible inhibitor of EGFR and has been approved for the treatment of Non-Small cell Lung Cancer (NSCLC) with sensitizing EGFR mutations by both the US Food and Drug Administration and the European Medicines Agency (Min *et al*., 2020). ESCC cell line is hypersensitive to Afatinib because it dramatically reduces the phosphorylation level of EGFR and induces apoptosis in ESCC cell line. In vivo, tumor growth is also dramatically decreased by Afatinib (Yu *et al*., 2016). Cabozantinib is an inhibitor of tyrosine kinases including MET, It is an oral multi-targeted small-molecule TKI, which targets VEGFR and MET. It has been approved in the USA to treat advanced renal cell carcinoma (RCC) and locally advanced or metastatic medullary thyroid cancer (MTC) (Al-Salama &Keating, 2016 and Fallahi *et al.,* 2015). Moreover Cabozantinib and R428 inhibit ESCC growth in cell and xenograft models (Pei-Wen *et al*., 2019). In this study we intend to concurrently inhibit EGFR and cMET using small molecular inhibitors and analyze their effect on the EC growth in vitro and in vivo. Taken together, these studies suggest that simultaneous inhibition of these two signaling pathways (EGFR and c-MET) could synergistically impede cell growth and could represent a potential therapeutic approach for EC treatment.

2. Review of Literature

2.1 International Level

Esophageal cancer is one of the common malignancies of the gastrointestinal tract (GIT). As per the World Health Organization (WHO), Globocon2018, esophageal cancer is the 7th most common cancer (3.2%) in the world, and 6th most common cause of cancer related mortality (5.3%). In the western world, the incidence of ESCC has declined steadily over the past three decades while AC has increased simultaneously. ADC is now the most dominant histological type in the western world (Vizcaino *et al*., 2002). The timing of the incidence of the two EC subtypes is different. The incidence of ADC in several Western countries is rising dramatically due to increased risk factors such as overweight and obesity (Pohl *et al*., 2010). On the contrary, the incidence of squamous cell cancer (SCC) in these countries is steadily declining due to reduced tobacco use and alcohol consumption. However, the incidence of ESCC in certain Asian countries, such as Taiwan, is likely to increase due to increased tobacco and alcohol consumption (Lu *et al*., 2010). Ten countries with the highest age-

standardized incidence rates for EC include Malawi (24.2 per 100,000) Turkmenistan (19.7 per 100,000) Mongolia (17.6 per 100,000) Kenya (17.6 per 100,000), Uganda (17.1 per 100,000), Lesotho (15.1 per 100,000), Tajikistan (14.7 per 100,000), Burundi (12.8 per 100,000), Bangladesh (12.7 per 100,000) and China (12.5 s per 100,000), respectively (Jamel *et al*., 2011). In the whole world, in 2012 the rate of new EC was 455800 cases, and the resulting deaths were reported to be 400200 (Wheeler & Reed, 2012). A significant change in the rate of incidence of EC indicates that various factors affect the increase in EC rates. On the other hand, most studies in this field focus more on clinical challenges and therapeutic approaches to EC management (Wouters *et al*., 2012 and Shivo *et al*., 2014). Racially, the incidence of ADC and ESCC is different between African- Americans, and Asians as compared to Caucasians. The incidence of ESCC in Caucasian men is 5 times higher than African Americans (Napier *et al*., 2014). The incidence of EC based on the geographical area is significantly different. In some countries, it is the second most common cancer and in some others one of the few common cancers. More importantly, the geographical distribution of EC differs according to subtypes; AC is more prevalent in developed countries, while ESCC is more prevalent in African and East Asian countries (Rustgi & El, 2014 and Arnold *et al*., 2015). Among the malignancies of the GIT, esophageal cancer accounts for 3.2%of all the newly diagnosed cancer patients in the world, this is behind colorectal (10.2%) and stomach (5.7%) cancer. EC accounts for about 5% (407,000 deaths) of all cancer deaths annually in the USA (Ferlay *et al*., 2015). In terms of regional distribution, the highest standardized incidence rate was observed in both sexes in East Asia (11 per 100,000), East Africa (9.7 per 100,000), South Africa (9.7 per 100,000), Asia (7.7 per 100,000) and Sub-Saharan Africa (4. 5 per 100,000), respectively. In the continental terms the highest incidence of EC was seen in Asia, Europe, Africa, North America, South America, and Oceania, respectively (Torre *et al*., 2015). Unfortunately, EC is often associated with an unfavorable prognosis for survival of 5 years, which varies from 4% to 40% based on the progression of the disease, with a total 5-year overall survival rate of 18% (Fitzmaurice *et al*., 2015). It has a very good prognosis in the case of early detection (Rafiemanesh *et al*., 2016). An increase in the incidence of EC with its recurrence has led to massive pressures on the health care system (Pakzad *et al*., 2016). In the United States, it is estimated that 17,290 EC cases are diagnosed each year and 15850 deaths due to the cancer are expected (Siegel *et al*., 2018). Conversely, the incidence of AC with its recurrence has led to massive pressure on the health care system, which is associated with an unfavorable prognosis for survival of 5 years varying from 4% to 40% based on disease progression with a total 5 year survival rate of

18%. Genomic alterations in cancer cells result in abnormalities of protein expression. This can give us an insight on whether certain drugs will be effective or not. A number of varieties of FDA approved drugs for EC have been marked or approved with resistance or improved outcomes. Despite significant improvements in multimodal therapies for EC patients who develop distant metastasis have dismal prognosis with limited cure.

2.2 National Level

ESCC and ADC are the two main histological types of EC. About 90% of the EC in the residents of Asia, Africa, and Eastern European countries is ESCC (Parkin *et al*., 2010). While the incidence of ESCC is decreasing, the incidences of ADC are increasing in western countries due to associated risk factors including smoking, alcohol, obesity, and gastro esophageal reflux disease. However, ESCC continues to be the major type of cancer in the Asian countries including India (Zhang *et al.,* 2012). As per the population-based cancer registry (PBCR) of India (National Center for Disease Informatics and Research, National Cancer Registry Program, Indian Council of Medical Research), the age-adjusted rate of esophageal cancer was highest in east Khasi Hill district of Meghalaya followed by Aizawl in Mizoram, irrespective of gender, for the latest period of 2012–2014. The age-adjusted rate of esophageal cancer was 71.2 for males and 33 for females (per 1,00,000) in east Khasi Hill District of India (National cancer Registry programme, 2012-2014). In India, EC is the 6^{th} most common cancer with incidence of 5.04%, 5^{th} most common cancer in males and 6^{th} most common cancer in females (WHO, Globocon 2018).

2.3 Regional Level

Among many states of India, Kashmir has the highest incidence and third highest in the world (Khuro *et al*., 2014). The Kashmir valley, in India's far north has been identified as EC hotspot (Dhar *et al*., 1993). While the alarming incidence in Kashmir has been linked to different dietary factors including use of hot salty tea, red chilies, meat, high-nitrate diet especially locally grown Brassica leaves (Mir & Dar, 2009), excessive use of tuibur (water filtrate of tobacco) and consumption of beetle leaves and tobacco in different forms also called smokeless tobacco (ST) are associated with development of EC (Kumar *et al*., 2001 and Ali *et al*., 2011). Esophageal cancer is a disease of advanced age, peaking in the seventh and eighth decades of life. Last but not the least affordability of treatment calls for an urgent and imperative action since the cost of cancer treatment is higher than other diseases. Due to the lack of validated target molecules, no targeted therapy for EC is yet approved. For this

reason, the identification of molecular targets and the rapid development of new therapeutic options remain critical for these patients.

3. Research Gap

The State of Jammu and Kashmir has the highest incidence of EC and third highest in the world. Since most of the EC patients don't respond to the current chemotherapeutic regimens due to intrinsic and acquired resistance and resulting in poor prognosis and low survival rates. In addition, while targeted therapies have increased response, but concurrent activation of signaling pathways result in treatment failure. Therefore, combination therapies as proposed in our study will not only inhibit growth and survival of EC cancer cells, it will also prevent development of recurrent tumors and improve therapy response.

4. Objectives

a) To investigate the effect of Afatinib, Cabozantinib and combination (AfCabCom) on esophageal cancer *in vitro*.
b) Investigate the molecular mechanism of AfCabCom action in EC.
c) To analyze the effect of Afatinib, Cabozantinib and (AfPCabCom) on EC *in vivo*.

5. Hypothesis

The use of EGFR inhibitors as monotherapy has yielded modest benefits, often associated with the development of resistance and refractory tumors. ESCC patients treated with anti EGFR drugs with low expression of c-MET have better OS than patients with overexpression of c-MET (Sheridan, 2014). Therefore, combination therapies as proposed in our study will not only inhibit growth and survival of EC cancer cells, it will also prevent development of recurrent tumors and improve therapy response. So, we hypothesized that "concurrent inhibition of both EGFR and c-MET pathways would synergistically inhibit the growth of EC and prevent the development of recurrent tumors.

6. Proposed Methodology

Objective 1: To investigate the effect of Afatinib, Cabozantinib and combination (AfCabCom) on esophageal cancer *in vitro*.

Proliferation assay

MTT assay protocol is adapted from literature methods. Briefly, EC cells will be seeded into 96-well plates ($1\text{-}5\times10^3$ cells/well) and will be allowed to attach overnight. Cells will be

treated with various concentrations of drug 24-72 hours after seeding. Media will be supplemented with 100 µl of MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reagent (c = 0.5 g/l) to each well and incubated for 30 min at 37°C. Thereafter MTT solution will be removed. After addition of 180 µl of DMSO the plates will be incubated for 15 min at 37°C to dissolve the formazan crystals. Absorbance readings will be measured at 560 nm with reference of 690 nm using microplate reader (Macha *et al*., 2010).

Clonogenic survival assay

For the clonogenic survival assay, EC cells (500) will be plated in each well of 6-well plate in 2.5 mL of culture medium. After 48 hours, cells will be treated with therapeutic agent at different concentrations; fresh medium with therapeutic agent will be replaced every 72 hours. After 15 days, colonies will be fixed in 4% formaldehyde and stained with 0.5% crystal violet (Jaime *et al*., 2020).

Boyden chamber assay

For transwell migration assay, cells are seeded in migration chambers in a 24 well plate with drug treatment. The 24 well plate is filled with media without drug and then observed cell migration after 72 hours; cells are fixed and washed, then observed under inverted microscope and checked for drug efficiency (Tomohiro *et al*., 2013).

Scratch wound assay

For scratch wound assay, cells will be seeded in a 6 well plate as a monolayer 70 to 80% confluence and after 24 h of growth remove media and make a scratch in the middle of the plate using fresh sterile pipette tip and given drug treatment for next 72 hours and observe the drug effects on migration (Tomohiro *et al*., 2013).

Quantitative *in situ* SA-β-gal assay

To analyze the effect of Afatinib, Cabozantinib and AfCabCom on EC cells, SA-β-gal assay will be done. Briefly, EC cells will be treated with Afatinib, Cabozantinib or combination for 72h and SA-β-Gal activity will be detected using X-gal (5-bromo-4-chloro-3-indolyl β-D-galactoside) staining at pH 6.0. Blue stained SA-β-gal-positive cells will be observed using light microscope and several representative fields (n = 6) will be randomly selected and quantified (Macha *et al.*, 2015).

Apoptosis assay

EC cells will be treated with chemotherapeutic agent for 24 and 48 hours. Apoptosis will be determined with the Annexin V-PE/7-AAD apoptosis kit (BD Biosciences, USA) as per the manufacturer's instructions. Briefly, cells will be trypsinized, washed twice with ice-cold PBS and the pellet will be resuspended in binding buffer (50 mM HEPES/NaOH, pH 7.4, 700

mM NaCl, 12.5 mM $CaCl_2$) containing Annexin/PI. After incubation for 15 minutes at room temperature in a light-protected area, the specimens will be quantified by FACS (Macha *et al*., 2010).

Objective 2: Investigate the molecular mechanism of AfCabCom action in EC. Western blot analysis:

Preparation of whole cell lysates from drug treated cells and immunoblot analysis will be done. Briefly, EC cells will be treated with Afatinib, Cabozantinib or with AfCabCom for 48h. Cells will be lysed in ice-cold RIPA (50mM Tris-HCl, 150mM NaCl, 1% NP-40, 0.5% sodium deoxycholate and 0.1% SDS) containing protease and phosphatase inhibitors. Equal amounts of protein will be loaded on 8-12% SDS PAGE and transferring protein on polyvinylidene difluoride (PVDF) membrane/ nitrocellulose, blots were blocked with 5% nonfat milk in PBS for2 h, followed by incubation with primary antibodies at 4°C overnight. After washing with PBS (0.1% tween), blots will be incubated with HRP-labeled appropriate secondary immunoglobulins for 1h. Blots will be developed using an enhanced chemiluminescence reagent kit (Amersham Pharmacia, Piscataway, NJ). B-actin will be used as a control for protein loading (Macha *et al*., 2010).

Objective 3: To analyze the effect of Afatinib, Cabozantinib and AfCabCom on EC *in vivo*.

In this objective, we will be injecting tumor cells subcutaneously into the nude mice on right and left thigh. Then allow the tumors to grow for 10-14 days. After this period, drug treatments are given to these mice (2-3 weeks) and the growth of the tumors will be examined.

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